

Antitumor effects of curcumin in pediatric rhabdomyosarcoma in combination with chemotherapy and phototherapy *in vitro*

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Abstract. Rhabdomyosarcoma (RMS), the most common pediatric soft tissue sarcoma, has an unfavorable outcome in advanced tumor stages with less than 30% failure-free survival. Curcumin (CUR) is a promising drug in complementary oncology with few side effects but proven efficacy in various adult oncological entities. The present study analyzed the effects of CUR on pediatric (RMS) cell lines *in vitro*. RMS cell lines (RD and RH30), and skeletal muscle cells (SKMC) were treated with different doses of CUR (1.5-30 μ M) alone, with phototherapy (PDT, 488 nm) or in combination with vincristine (VCR) or dactinomycin (DAC). MTT assays were used for analysis of RMS tumor cell viability. Clonal cell growth was assessed via colony forming assays and migration of the cells was analyzed with scratch tests. Annexin V staining was used to determine apoptosis in flow cytometry. Possible RMS resistance towards CUR after long-term treatment was analyzed with MTT assays. CUR decreased cell viability in all assessed RMS cell lines in a concentration-dependent manner with IC_{50} =14-20 μ M. CUR enhanced the effects of the cytotoxic drugs VCR or DAC, and led to reduced migration and increased cell apoptosis. In combination with PDT, CUR decreased the cell viability in minute quantities with up to a 10-fold lower IC_{50} than without PDT. CUR effectively inhibited the malignant properties of pediatric RMS cells and should be focused on as a useful additional agent in standard chemotherapy of RMS in children.

Introduction

Rhabdomyosarcoma (RMS) is one of the three most frequent extracranial solid tumors in childhood, directly after nephroblastoma and malignant bone tumors. In the group of soft tissue and other extraosseous sarcomas it represents the most frequent type (1). The outcome is mainly influenced by tumor localization, histological subtype and molecular characterization, IRS stage, and age at presentation (2). Embryonal and alveolar RMS are the most common histological subtypes, while botryoid and spindle cell variants are less common. The embryonal RMS subtype has a favorable 5-year survival rate compared to the alveolar subtype. The alveolar RMS subtype is in 80% characterized by chromosomal translocations t(2;13) or t(1;13) resulting in FOXO1 fusion genes (60% PAX3, 20% PAX7); FOXO1 fusion genes in embryonal RMS are rare. According to the Children Oncology Group (COG), RMS with FOXO1 fusion genes correlate with a worse prognosis (3). The spindle cell/sclerosing subtype is a markedly aggressive RMS accounting for 5-10% of all RMS cases with a wide age distribution (4). Using the information about the pre-treatment staging (TNM, based on anatomic site tumor-node-metastasis), the extent of the disease, after surgical resection (clinical group), primary tumor site, and histology/fusion status, a risk stratification system has been established with an accurate prediction of patient outcomes dividing the patients in low-, intermediate-, and high-risk groups (5). Multimodality treatment includes chemotherapy and surgery with or without radiotherapy (6). The backbone of most chemotherapeutic treatment protocols is a combination of a three-drug regimen with vincristine (VCR), dactinomycin (DAC), and cyclophosphamide (VAC); eventually low-risk patients are treated only with a two-drug regimen of VCR and DAC (6). Even in high-risk and metastatic RMS patients, VAC remains the chemotherapy with the highest effects, and treatment regimens with additional or substitute components could not rule out the benefits of VAC therapy (7). Even in cases with relapse, VCR is also part of common therapeutic regimens (8). VCR as well as other similar vinca alkaloid agents may cause acute and long-term damage to peripheral nerves. As a consequence, severe neuropathy leads to dose reduction or even cessation of therapy; in a great number of patients significant long-term impairments persist after completion of treatment (9).

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Abbreviations: CUR, curcumin; DAC, dactinomycin; PDT, phototherapy; RMS, rhabdomyosarcoma; VCR, vincristine

Key words: curcumin, rhabdomyosarcoma, phototherapy, pediatric solid tumor

Since 1970, the overall survival rate of RMS has increased from 25 to 70% in 1990. However, compared to a high failure-free survival (FFS) of 88% in the low-risk group, and 55-76% in the intermediate group, the outcome of the high-risk group remains poor with only 10-30% FFS and has not improved during the last 30 years (10). This is mainly due to the relatively low incidence of the disease and even lower rates of high-risk cases. The result is a limited number and timing of new clinical trials that tests new potential treatments. Preclinical efforts to identify potential new treatments with *in vitro* cell-line research are an important cornerstone of initiating further clinical trials with promising new drugs. Recent attempts include investigations of natural or synthetic chemopreventive small molecules and drugs (11).

One of the most extensively studied phytochemicals of complementary oncology is curcumin (CUR), a yellow-orange dye derived from the rhizome of the plant *Curcuma longa*. In tumors it induces apoptosis, inhibits cell proliferation, and efficiently affects several pathways associated with cancer stem cell self-renewal (12). Moreover, it facilitates absorption of radiation between 350-500 nm and causes oxygen-dependent phototoxicity (13). Since more than three decades, basic research has revealed effects on tumors; it has been analyzed in multiple Phase-II and -III studies in adult patients with varying malignant tumors as a supplement treatment in high-risk cases (14). CUR should be considered for pediatric oncological therapy due to its tolerability and minimal side effects (15). Furthermore, there is some evidence that curcumin significantly attenuates the neurotoxic side effects of VCR (16,17). The low bioavailability of native CUR was recently overcome by micellar galenics (18). In an *in vivo* model of pediatric hepatocellular carcinoma in mice we could demonstrate both, the antitumor effects on orthotopic tumors in a combination therapy of CUR and cisplatin and relevant CUR concentrations in blood and organs after oral administration of micellar CUR (19). Furthermore, additional phototherapy (PDT) amplified the inhibitory effect of CUR on hepatoma cells *in vitro* up to 20-fold (13). Few studies have elucidated the effects of CUR on sarcoma, especially RMS cells (20,21).

The present study explored the effects of CUR alone or in combination with the cytotoxic drugs VCR, or DAC or with PDT on cell viability, proliferation and migration in RMS cells *in vitro*.

Materials and methods

Drugs and chemicals. The native CUR powder of the plant *Curcuma longa* Linn. used in all formulations contained 82% CUR, 16% demethoxycurcumin (DMC), and 2% bis-demethoxycurcumin (BDMC) and was purchased by Aquanova. The stock solution consisted of 15 mg CUR dissolved in 1 ml DMSO. The final maximal concentration of CUR was 30 μ M, and the final concentrations of DMSO used in experiments were <0.0007% (v/v). Control groups were treated with 0.01% DMSO. The cytotoxic drug VCR was purchased from Teva, and the stock solution contained 1 mg VCR sulfate per ml. The cytotoxic drug dactinomycin (DAC; Cosmegen®-Lyevac) was purchased from MSD Sharp & Dome GmbH, and the stock solution contained 500 mg DAC with 20 mg mannitol. Stock solutions of VCR, and DAC were further diluted in DMEM.

Cell lines and culture conditions. The embryonal RMS (ERMS) cell line RD was purchased from ATCC. It was initially obtained from a seven-year old girl with relapsing pelvic RMS. The cells have 51-hyperdiploid chromosomes and show amplification of MYC oncogene, Q61H mutation of NRAS, and homozygous mutations of TP53. RD cells are one of the most commonly used cell lines in RMS research (22,23). The alveolar RMS (ARMS) cell line RH30 expressing the Pax3/FOXO1 fusion protein secondary to the t(2;13)(q35;q14) translocation was obtained from DSMZ. It was initially isolated from a bone metastasis of a 17-year old boy with untreated RMS (22).

Normal human skeletal muscle cells (SKMC) from adult donors were purchased from PromoCell GmbH. All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) with GlutaMAX, 4.5 g/l D-glucose (Gibco, Life Technologies; Thermo Fisher Scientific, Inc.) supplemented with 10% FCS (Biochrom GmbH) and 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. No additional mitogen was added, except FCS, neither in the cell culture nor in the experiments. All cell cultures were mycoplasma species-negative. For subculturing, cells were detached from the culture surface using 0.05% Trypsin-EDTA (Gibco Life Technologies; Thermo Fisher Scientific, Inc.).

Viability assays. Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT) assay (AppliChem GmbH) as previously described (24). All assays were performed at least three times independently in quadruplicates. Percentages of viability were calculated through normalization between background of cultures without cells and untreated cultures as control experiments. Dose-dependent viability curves were computed by sigmoidal curves with variable slopes to determine IC₅₀ values.

Tumor cells (1x10⁴ cells/100 μ l) were cultured in 96-well plates. After 24 h of incubation the supernatant was exchanged, and therapeutic agents were added in increasing concentrations: native CUR (5-30 μ M), VCR (0.3-80 nM) or DAC (0.1-13 nM); cells were incubated for 48 or 72 h. In another series of experiments, a combination treatment with CUR and VCR, or CUR and DAC was added for 48 or 72 h. Furthermore, the effects of PDT were analyzed: cells were treated with CUR for 48 h in concentrations of approximately 10-fold lower than the IC₅₀. Cells were washed and 1 h after CUR treatment PDT was performed (lambda 488 nm, 5 sec; 300 W xenon short-arc lamp; Karl Storz SE & Co. KG).

For the analysis of potential CUR resistance, cells were incubated with increasing CUR concentrations as a long-term treatment model [start concentration 0.5 μ g/ml (1.357 μ M); increase steps 0.5 μ g/ml (1.357 μ M); and end concentration 8 μ g/ml (21.72 μ M)]. After the end concentration was reached, cells were cultured without CUR for 72 h. Subsequently, after another 72-h CUR incubation, an MTT-assay was performed comparing initial untreated cells with cells after long-term treatment.

Evaluation of drug interaction. To analyze the inhibitory effect of drug combinations, the coefficient of drug interaction (CDI) was calculated. This was performed according to the Bliss Independence model, which is considered one of the most

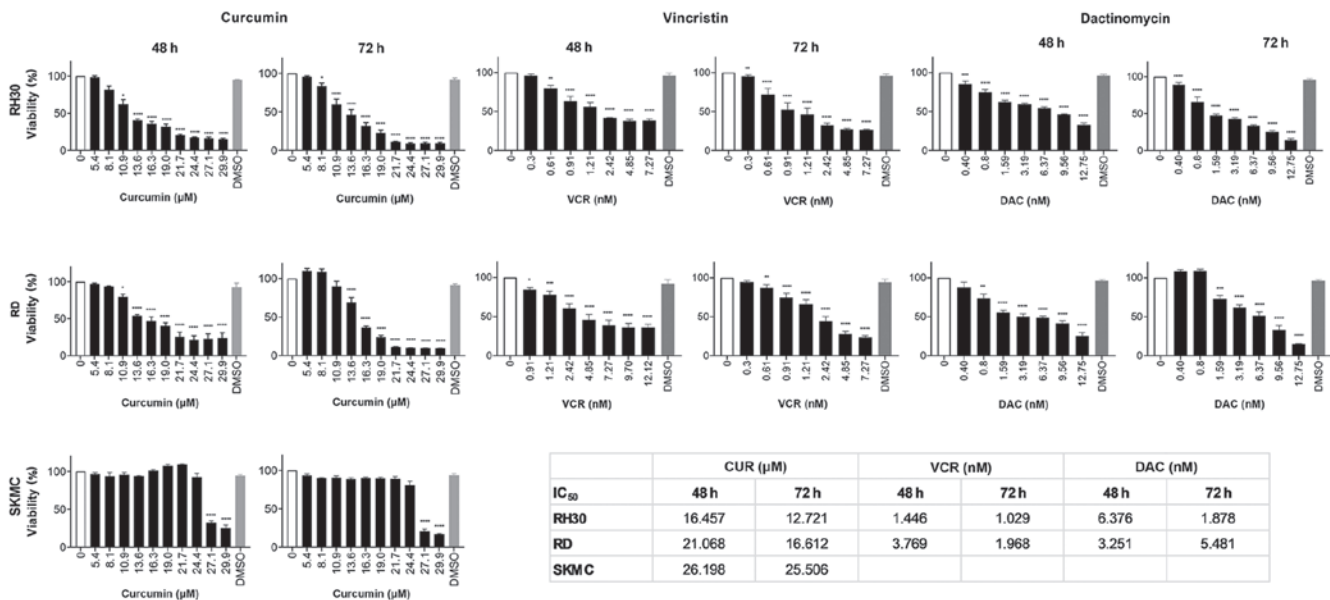


Figure 1. Effect of CUR, VCR or DAC on the viability of RH30 and RD cells (and SKMC cells in case of CUR). Arithmetic means \pm SEM (n=6) of the relative number of viable cells following a 48 or 72 h incubation, respectively, in the presence of increasing concentrations of the therapeutic agents. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 indicates statistical significance compared to control (0 μM). The IC₅₀ values are presented in the table. One-way ANOVA with Dunnett's test was performed. CUR, curcumin; VCR, vincristine; DAC, dactinomycin; SKMC, human skeletal muscle cells.

popular models to assess the combined effects of drugs (25). CDI was calculated as following: $\text{CDI} = (\text{A} + \text{B} - \text{A} \times \text{B}) / \text{AB}$. AB is the ratio of the absorbance in the combination drugs to control; A or B is the ratio of the absorbance of the single agent group to the control group. CDI values <1, =1, or >1 indicated that the drugs were synergistic, additive, or antagonistic, respectively. Inhibition rates obtained from the MTT assays were used for these calculations.

Clonogenic assays. RMS cells were plated in 6-well-plates with 750 cells/well and treated with increasing CUR concentrations. After 72 h cells were washed with PBS and fresh medium (without treatment) was added. After 14 days, subsequent grown cell colonies were washed with PBS and fixed twice in methanol for 5 min, at room temperature (RT). Visualization of fixed cell colonies was achieved by incubation with 1% (w/v) crystal violet for 30 min for RD cells and 120 min for RH30 cells, at RT. Visible colonies consisting of >50 cells were counted. Dividing the number of colonies by the number of plated cells and multiplying by 100 yielded the colony formation rate according to Franken *et al* (26). Images were captured using phase-contrast microscope Zeiss Axiovert 135 microscope (original magnification, x5; Carl Zeiss Microscopy GmbH).

Cell migration. A wound healing assay was performed to assess the migratory inhibition effect of CUR and of CUR in combination with VCR. Each test was performed at least in triplicates. Cells were grown in the presence of the complete growth media (FCS >5%) to 90% confluence and subdivided into a 6-well cell culture plate (1x10⁶/well). A scratch across each well was produced using a 10-100 μl pipette tip. Cellular debris was gently washed with PBS. The growth media was exchanged, CUR, and/or VCR were added, and migration potential was estimated for RD cells after 0, 24 and 30 h, and

for RH30 cells after 0, 24, 30 and 48 h. The cell migration area was quantified by analyzing images, and images were acquired at the defined time-points using a phase-contrast microscope Zeiss Axiovert 135 microscope (x5, magnification), and AxioVision software LE64, 4.9.1 (Carl Zeiss Microscopy GmbH). The percentage of cell migration was calculated.

Flow cytometry. Apoptosis was analyzed using flow cytometry with Annexin V staining with allophycocyanin (APC) conjugation (BD Biosciences) after single treatment with the following concentrations below the respective IC₅₀: CUR 10.86 μM , or 13.57 μM for RD cells, and 13.57 μM , or 16.29 μM for RH30 cells; VCR 0.91 nM, DAC 1.20 nM) and combination treatment (CUR and VCR, CUR and DAC). After 48 h, cells were detached from the culture surface using 0.05% Trypsin-EDTA (Gibco Life Technologies; Thermo Fisher Scientific, Inc.) and washed with Annexin binding buffer (1:10). The staining with APC-conjugated Annexin V was performed according to a standard BD Bioscience protocol. The staining with propidium iodide was interfered by the fluorescence of intracellular CUR in the PE channel and thus could not be performed (Fig. S1). Samples were analyzed on a FACSCanto II flow cytometer and evaluated with BD FACS Diva software Version 8.0 (BD Biosciences).

Statistical analyses. Data analysis was carried out using GraphPad Prism 8.4.0.00 (GraphPad Software, Inc.). In MTT, the IC₅₀ was calculated from the sigmoid dose-response curves with variable slopes. In fluorescence measurements, LOG half maximal effective concentration (EC₅₀)-values were calculated on the basis of sigmoidal dose-response curves with variable slopes. The obtained curves on RMS cells for each treatment were compared to their IC₅₀ or LOGEC₅₀ values, and the slope and the P-value were determined with 95% confidence intervals (CI).

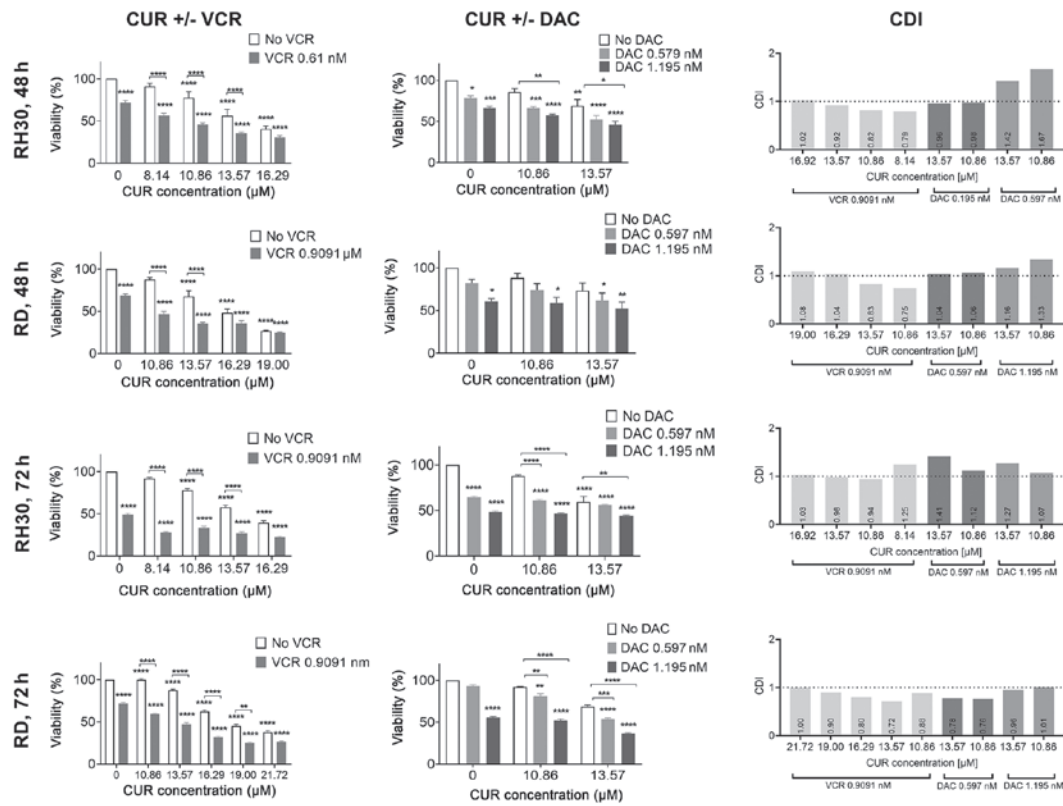


Figure 2. Effect of combination therapies of CUR and VCR, or CUR and DAC on cell viability. Left rows, effect of combination therapy with CUR and VCR. Middle rows, effect of combination therapy with CUR and DAC. Arithmetic means \pm SEM ($n=6$) of the relative number of viable RMS cells following a 48 or 72 h incubation in the presence of 13.57 μ M CUR along with VCR (RH30 and RD, 0.61 or 0.91 nM), or DAC (RH30 and RD, 0.597 or 1.195 nM). Two-way ANOVA with Bonferroni's multiple comparison test was performed. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ and **** $P<0.0001$ indicates statistical significance compared to the control (simple asterisks) or compared to each other (asterisks with a square bracket). Right rows, CDI according to Bliss Independence. Values below 1 (broken line) indicate synergism, values above 1 indicate antagonism, and 1 indicates additive effects. CUR, curcumin; VCR, vincristine; DAC, dactinomycin; RMS, rhabdomyosarcoma; CDI, coefficients of drug interaction.

Comparison of two regression curves was performed by an F-test and a significant difference was obtained at P -values <0.05 . All numeric data are expressed as arithmetic means and standard error of means (SEM).

All data were tested for significance with either a one-way ANOVA (post hoc Dunnett's multiple comparison test) or two-way ANOVA (post hoc Bonferroni's multiple comparison test).

Results

Effects of curcumin on cell viability and evaluation of drug interaction. As revealed in Fig. 1, CUR decreased cell viability in all assessed cell lines in a concentration-dependent manner. The IC_{50} values were lower after 72 h of incubation than after 48 h. Longer incubation of CUR for more than 72 h did not result in a further loss of viability in any of the cell lines (data not shown). The viability of SKMC was not impaired by CUR, only high concentrations of $>25 \mu$ M led to a decrease. The decrease of cell viability by VCR and DAC was also concentration-dependent with IC_{50} 1-14 nM for VCR, and IC_{50} 1-6 nM for DAC. As VCR and DAC are routinely used in chemotherapeutic regimens for decades, these substances were not assessed for SKMC cells.

A further series of experiments evaluated the impact of combined incubation of CUR either with VCR or DAC and

revealed increased effects indicating that CUR may have some chemo-sparing effects (Fig. 2). The calculated coefficients of drug interaction revealed synergistic effects of CUR and VCR in RH30 and RD cells. The combination of CUR with DAC had an antagonistic effect in RD cells and a synergistic effect in RH30 cells (Fig. 2).

Effects of CUR and PDT on RMS cell viability. The combination of CUR and PDT had higher potency than CUR alone. IC_{50} values were decreased compared to treatment without PDT (RH30 cells, 1.67 μ M; RD cells, 1.93 μ M; Fig. 3). In SKMC cells the combination of CUR with PDT did not significantly decrease cell viability (Fig. 3C).

Effects of pretreatment and resistance to CUR. Another series of experiments explored whether a long-term pretreatment of the cells with increasing CUR concentrations resulted in any resistance to CUR. While the viability of pretreated cells was less decreased with CUR in high doses of 21.72 μ M in RD, and 10.86 μ M in RH30 cells, with a significant difference obtained with one-way ANOVA test, in a comparison of the IC_{50} , no significance was obtained concerning the IC_{50} of RD cells untreated, IC_{50} 11.85 μ M; RD cells pretreated, IC_{50} 14.11 μ M; RH30 cells untreated, IC_{50} 10.76 μ M; and RH30 cells pretreated, IC_{50} 11.19 μ M. (Fig. 4).

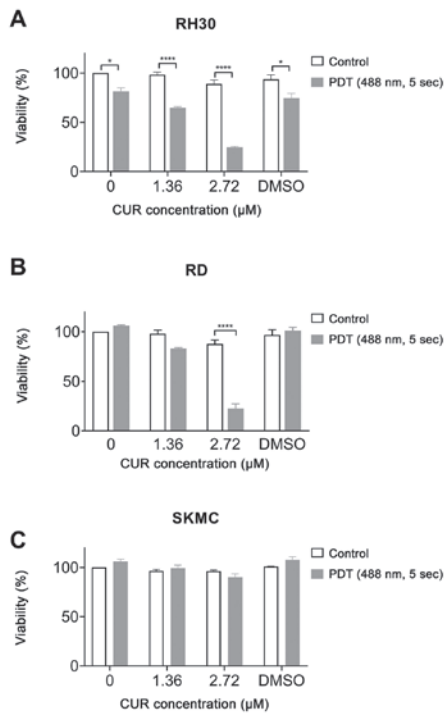


Figure 3. (A-C) Effect of PDT with 488 nm blue light for 5 sec and CUR incubation on cell viability. Arithmetic means \pm SEM (n=3) of the relative number of viable (A) RD, (B) RH30 and (C) SKMC cells following irradiation with blue light for 5 sec after a 48 h incubation with low doses of CUR. Two-way ANOVA and Bonferroni tests were performed. *P<0.05 and ****P<0.0001 indicates statistical significance. PDT, phototherapy; CUR, curcumin; SKMC, human skeletal muscle cells.

Effects of CUR on colony formation of RMS cells. Since the colony forming potential of a single tumor cell is a marker of its malignant potential, the effect of CUR on colony formation was analyzed. Even concentrations that were markedly lower than the respective IC₅₀ led to a significant decrease of colonies: in RD cells 2.72 μ M and in RH30 5.43 μ M (Fig. 5).

Effects of CUR and drug combinations on RMS cell migration. One aspect of the increased effects of CUR with VCR was revealed in the migration assays. The migration abilities of the RMS cells RH30 and RD were inhibited more effectively by CUR and VCR in combination with concentrations below the IC₅₀ compared to VCR or CUR alone (Figs. 6-8). Notably, CUR alone did not significantly decrease the migration at the IC₅₀ value in RD cells, while it did so in the RH30 cells. In contrast, VCR decreased the migration as a single drug in RD cells, but not in RH30 cells. The cells in the cell migration assay were supplemented with >5% FCS; lower concentrations led to a significant increase of apoptotic cells and an insufficient healing of the scratch during 48 h. Furthermore, the long doubling times for the cells (RD cells 23 h, RH30 cells 37 h) (27) also minimize a falsification of the migration assay by the use of FCS >5%.

Effects of CUR and drug combinations on apoptosis in RMS cells. Another important aspect of the increased effects in the combination therapies was revealed by apoptosis assays (Fig. 9). With VCR or DAC alone, nearly no apoptosis was detectable. With CUR alone, apoptosis was induced only by

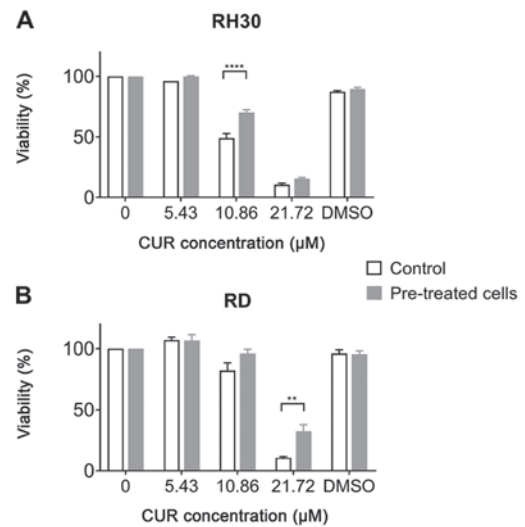


Figure 4. Effect of CUR on the viability of RMS cells after long-term pre-treatment with increasing doses of CUR. Arithmetic means \pm SEM (n=3) of the relative number of viable (A) RH30 and (B) RD cells following incubation CUR in increasing concentrations. Two-way ANOVA with Bonferroni's test was performed. **P<0.01 and ****P<0.0001 indicates statistical significance. CUR, curcumin; RMS, rhabdomyosarcoma.

high doses near the respective IC₅₀ (RD, 13.57 μ M; RH30, 16.3 μ M). This effect was markedly enhanced in the combination treatment of CUR along with VCR, or DAC.

Discussion

Soft tissue sarcomas represent less than 6% of childhood malignancies including the subgroup of RMS accounting for 4.5%. Similar to other childhood malignancies, the overall survival of RMS patients has improved from less than 20% to more than 80% since 1950, but the outcome of patients with advanced tumor stages remains poor (1). Children with high-risk RMS tumors represent approximately 15% of patients with initially diagnosed RMS (5). These relatively low numbers hamper randomization and thus are a limiting effect of further optimization studies. In an attempt to increase survival even in these high-risk tumors new therapeutic strategies are required. Well-tolerated phytotherapeutics with additional chemotherapeutic-sparing effects are favorable. CUR has been in the focus of medical research for more than three decades and meets these criteria perfectly. In addition to varying anti-inflammatory and neuroprotective properties, its demonstrated antineoplastic potential has been summarized in several reviews (28-30). However, little research has been conducted on the effect of CUR on pediatric solid tumors.

The present study evaluated the anticancer activity of CUR on RMS cells. Its effect on cell viability, proliferation, colony forming, and apoptosis was analyzed and was compared to the effects of the standard chemotherapeutic agents, VCR and DAC.

In the recent study, it was revealed, for the first time to the best of our knowledge, that treatment of the RMS cell lines RH30 and RD with CUR significantly decreased the viability, clonogenic ability, and capacity to migrate of RMS cells. In a prior study, high concentrations of CUR (20 μ M) only slightly reduced the viability of one liposarcoma cell line and one synovial sarcoma

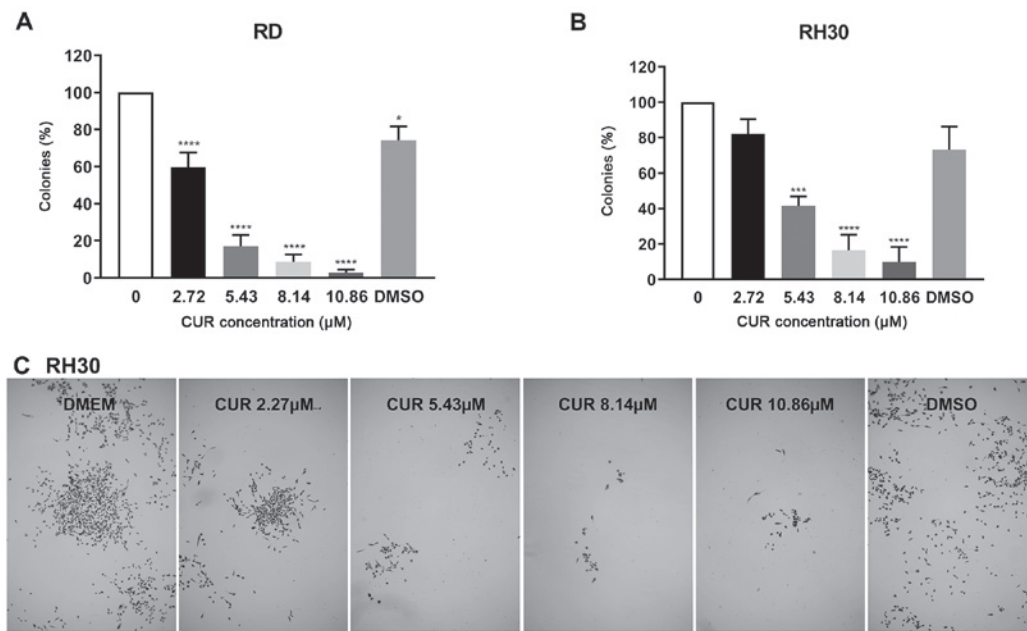


Figure 5. Effect of CUR on colony forming ability of RH30 and RD cells. (A and B) Arithmetic means \pm SEM ($n=4$) of the percentage of evolving colonies of (A) RD and (B) RH30 cells following a 72 h incubation in the presence of 2.72, 5.43, 8.14 and 10.86 μM CUR relative to the colonies in the absence of CUR (white bars). One-way ANOVA with Dunnett's test was performed. * $P<0.05$, **** $P<0.0001$ and *** $P<0.001$ indicates statistical significance compared to control (0 μM). (C) Representative images of colonies of RH30 cells. CUR, curcumin.

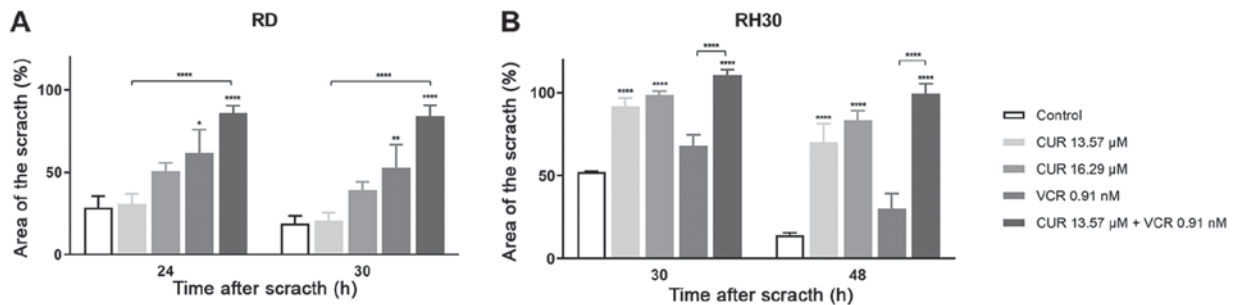


Figure 6. Effect of combination therapy on migration. (A and B) Arithmetic means \pm SEM ($n=8$) of the percentage of migrated (A) RD and (B) RH30 cells after incubation with CUR (13.57 and 16.29 μM), VCR (0.91 nM) and a combination of CUR (13.57 μM) with VCR (0.91 nM). Two-way ANOVA and Bonferroni tests were performed. * $P<0.05$, ** $P<0.01$, and **** $P<0.0001$ indicates statistical significance. CUR, curcumin; VCR, vincristine.

cell line, while in several other sarcoma cell lines CUR treatment had no effect on cell viability (21). The present study revealed that low concentrations of CUR (12–15 μM) resulted in a 50% viability reduction in pediatric RD and RH30 cells. In SKMC, CUR had lower potency ($\text{IC}_{50} > 25 \mu\text{M}$). Another study used cell proliferation assays and obtained even lower IC_{50} values in RMS cell lines (RH30 5.1 μM and RH1 2.7 μM) (31).

RD cells (embryonal RMS cells), one of the most commonly used cell lines in RMS research, have been demonstrated to undergo growth inhibition when treated with VCR and VAC, but not DAC, and doxorubicin in *in vivo* mouse models (32). The cell line RH30 represents the alveolar RMS subtype with FOXO1 fusion. In RH30 xenografts, VCR led to tumor regression while DAC or doxorubicin induced no growth inhibition (32). In the present *in vitro* results, RD and RH30 cell lines were inhibited only by high concentrations of DAC, but CUR resulted in a viability decrease in all RMS cell lines. The highest reported peak serum concentration of oral administered CUR (micellar galenics) in a human trial

was $\sim 3.2 \mu\text{M}$ (18). In our previous study involving mice we measured peak serum concentrations $\sim 2\text{--}3 \mu\text{M}$ but also analyzed the concentrations of curcumin in inner organs and liver tumors and the highest concentration in the lungs was $\sim 0.008 \mu\text{M}/\text{kg}$ (19). Compared to the required IC_{50} in cell culture, the achievable serum concentrations are markedly low. On the other hand, these comparatively low serum concentrations, in a murine tumor model for hepatocellular carcinoma, were able to induce a significant inhibitory effect on tumor growth (19). To the best of our knowledge, no investigations concerning CUR concentrations in muscle cells or even muscle tumors exist. In a recent study we analyzed the effects of CUR on normal SKMC and observed the same phenomena of increased viability in concentrations of $\sim 20 \mu\text{M}$, indicating a good conservation of the surrounding muscular tissue *in vivo*. This was described before in other studies with fibroblasts; CUR below a concentration of 25 μM led to an increase in viability, while concentrations higher than 25 μM had an inhibitory effect (21,33).

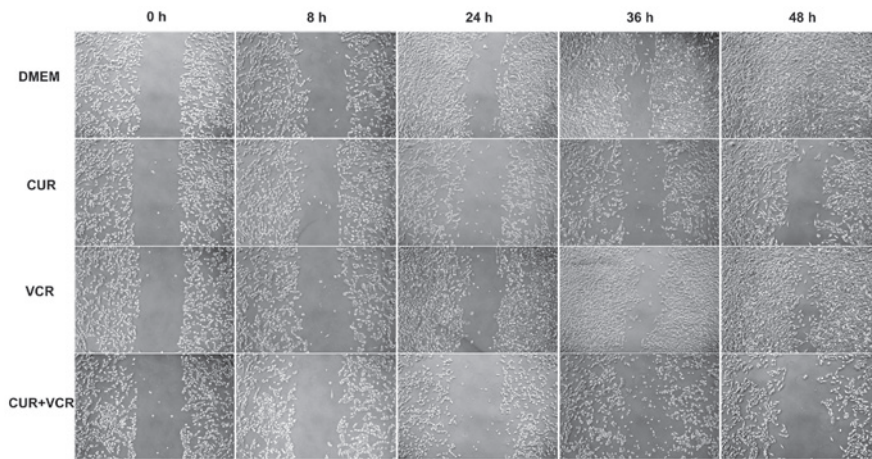


Figure 7. Effects of VCR and CUR on migration of RD cells. Representative histological panels. CUR in single therapy at $16.29 \mu\text{M}$, and in combination therapy with VCR at $13.57 \mu\text{M}$ at different time-points (magnification, x5). VCR, vincristine; CUR, curcumin.

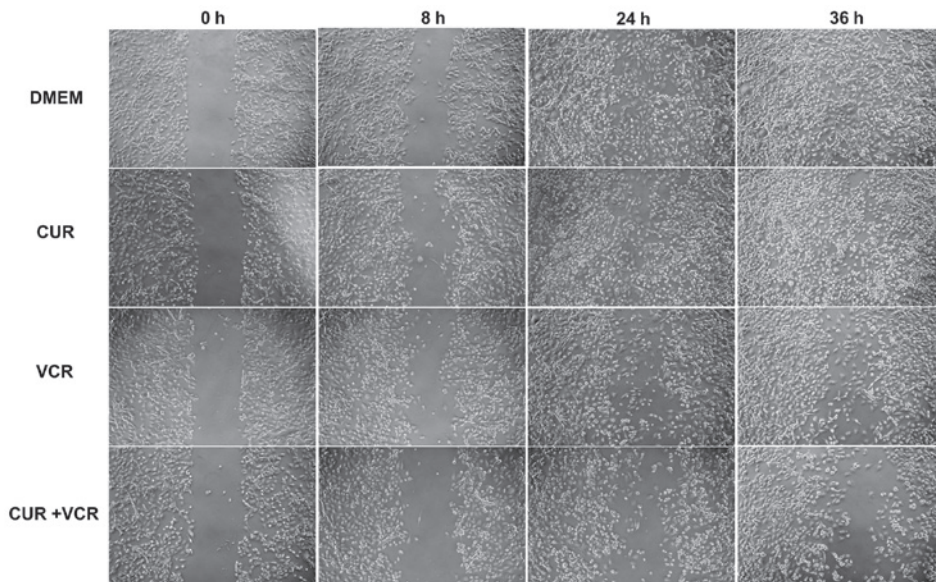


Figure 8. Effects of VCR and CUR on migration of RH30 cells. Representative histological panels. CUR in single therapy at $16.29 \mu\text{M}$, in combination therapy with VCR at $13.57 \mu\text{M}$ at different time-points (magnification, x5). VCR, vincristine; CUR, curcumin.

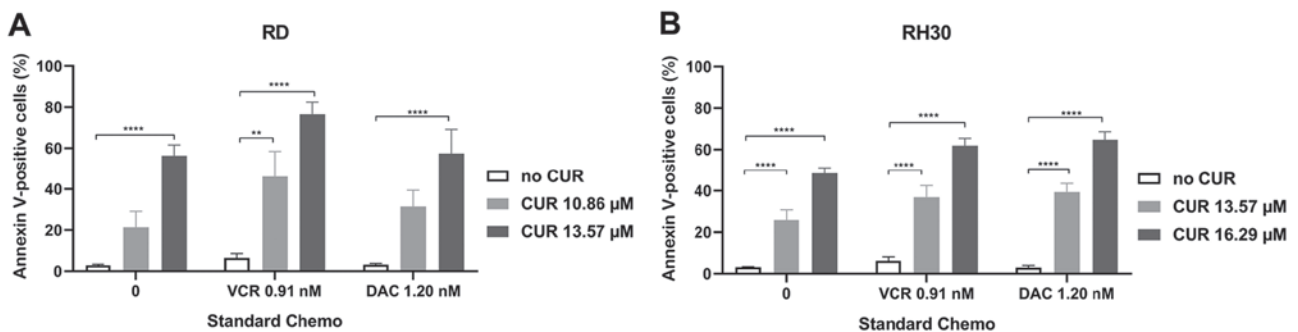


Figure 9. Effect of combination therapies on apoptosis. Arithmetic means \pm SEM (n=4) of the number of Annexin V-positive cells after incubation with different concentrations of CUR w/o VCR (0.91 nM) or DAC (1.2 nM) of (A) RD or (B) RH30 cells. Two-way ANOVA with Bonferroni's test was performed. * $P < 0.05$ and **** $P < 0.0001$ indicates statistical significance. CUR, curcumin; VCR, vincristine; DAC, dactinomycin.

Moreover, in the present study CUR enhanced the sensitivity of RMS cells to the cytotoxic drugs VCR and DAC. This enhanced sensitivity affected cell viability, capacity to

migrate, and apoptosis. Notably, the colony forming potential was significantly decreased even with low concentrations of CUR. As the IC_{50} for cell viability describes only one main

aspect of the effect of a drug, even drug concentrations below the IC_{50} value may have an impact on cells. It was demonstrated in the present study that CUR decreased the ability of colony formation of RD cells in concentrations 10-fold lower than the IC_{50} value and of RH30 cells in concentrations 3-fold lower than the IC_{50} value. This fact may be indicative of the ability of CUR to inhibit the self-renewal properties of RMS cells. Similar findings of low-dose CUR effects on the colony-forming potential of glioblastoma stem cells have been reported (34). Moreover, in combination with VCR, CUR in doses beyond the IC_{50} values inhibited migration of RD cells, and CUR in doses of the IC_{50} inhibited migration of RH30 cells, which may be interpreted as an effect on metastatic potential, since most patients with relapsing RMS suffer from local recurrence due to invasive disease.

One of the major causes of failure in treating children with RMS is the development of resistance to chemotherapy. Especially in advanced and relapsed cases several of these tumors are only initially chemosensitive, but may acquire multidrug resistance during chemotherapy (35). While the enhanced expression of multidrug resistance-related proteins including MDR1, MRP, or LPR may lead to an enhanced efflux of chemotherapeutic agents out of the tumor cells (36), the blocking of apoptosis signaling is another important characteristic feature of RMS that has been associated with poor treatment response (37). Herein it was revealed that the single treatment of VCR or DAC did not induce apoptosis in RMS cells, but high doses of CUR did. Furthermore, the combination of CUR with VCR, or DAC resulted in a further increase of apoptosis. Apoptosis induction as one effect of the antineoplastic potential of CUR has been described in different solid tumors, as well as in RMS cell lines, RH30 and RH1 (31,38-41). While Beevers *et al* described the pro-apoptotic effects of CUR at a concentration of 20 μ M (31), we revealed similar and dose-dependent effects in concentrations of 13.57 μ M in RH30 cells and 10.86 μ M in RD cells. A limitation of our apoptosis assay is the limited usability of the necrosis marker propidium iodide due to the overlapping emissions of CUR and the PI dye in the PE channel. In the present study, it was also demonstrated that PDT (480 nm) of RMS cells shortly after CUR treatment amplified the cytotoxicity of CUR in both assessed RMS tumor cell lines but not in normal SKMC. Transferring these findings in an *in vivo* model, after tumor resection, the PDT of the tumor bed with blue light shortly after CUR administration may result in a further destruction of remaining invisible micrometastases without any harm to surrounding tissues. An orthotopic *in vivo* model to explore the impact of CUR and PDT on surrounding normal tissue is currently under development.

Although the present study did not focus on the pathways involved in the inhibitory effects of CUR on RMS cells, it did indicate that the additive use of CUR should be a therapeutic option in the treatment of RMS. A further limitation of the present study was the fact, that we used only two RMS cell lines and not more fusion-negative and fusion-positive cell lines. On the other hand, there are hardly any studies on solid tumors in children treated with CUR, thus the present results have an important impact as a basis for further investigations. Furthermore, we hope to be able to add *in vivo* assessment to our current knowledge in a future model with orthotopic RMS tumors in mice.

In summary, it was demonstrated that CUR effectively decreased the viability of RMS cells in a dose-dependent manner. In combination with VCR or DAC, CUR inhibited cell viability in an additive and in some combinations even in a synergistic way. In combination with PDT, markedly low doses far beyond the IC_{50} values of CUR decreased RMS cell viability. Additionally, it was revealed that low doses of CUR inhibited important abilities of RMS cells including colony formation capacity. Moreover, pretreatment of RMS cells with CUR did not lead to signs of CUR resistance. The present findings indicated that CUR may be an effective and safe additional chemotherapeutic drug for the treatment of RMS in children.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

VE, ES, and JF conceived the study. CS, VE, ES, and NB acquired and analyzed the data. All authors drafted the work and revised it, and provided their final approval of the version to be published. All authors agree to be accountable for all aspects of the work.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no financial or non-financial competing interests.

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